

# **The Development of a Simple and Highly Sensitive Assay for Measuring Lipid Oxidation**

An Honors Thesis (HONRS 499)

by

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A handwritten signature in black ink, appearing to read "Scott Pattison", with a long horizontal flourish extending to the right.

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Abstract:

Oxidation is an essential process that is carried out in all cells. Free radicals are a by-product of this process. Lipid oxidation, caused by damage by non-intercepted free radicals, is the cause of many degenerative diseases. Currently, all existing methods for measuring lipid oxidation have complications. These shortcomings, including lengthy procedures, slow reactions, strict experimental conditions, and low sensitivity, make the development of a new assay desirable. A new method was developed that overcame all of these shortcomings. Tertiary butyl hydroperoxide (t-BuOOH) was used to simulate an oxidized membrane. Diphenyl-1-pyrenylphosphine (DPPP) was used to measure the oxidation by reacting with t-BuOOH and becoming diphenyl-1-pyrenylphosphine oxide (DPPPO), which fluoresces. This fluorescence was measured by using a High Performance Liquid Chromatography instrument for Flow Injection Analysis. An  $\text{Fe}^{3+}$ /pyridine complex was used as a catalyst. During the process of developing this method, new complications arose, such as signal depression and reaction with molecular oxygen, but they were overcome. In the end, a highly sensitive and simple assay for measuring lipid oxidation had been developed.

Acknowledgements:

I would like to thank Dr. Pattison for the opportunity to take part in this research project. Working with Dr. Pattison throughout the last semester was an enjoyable experience through which I learned a lot and gained valuable experience. I would also like to thank the Ball State Chemistry Department for the unique opportunity to do research as an undergraduate. I must also thank Laura Kenkel who worked with me on this project throughout the last semester. Her knowledge and previous experience with this project was a great help as we worked together to continue the research this semester. The knowledge and experience I gained from this project has been invaluable and it will be very useful in my future career.

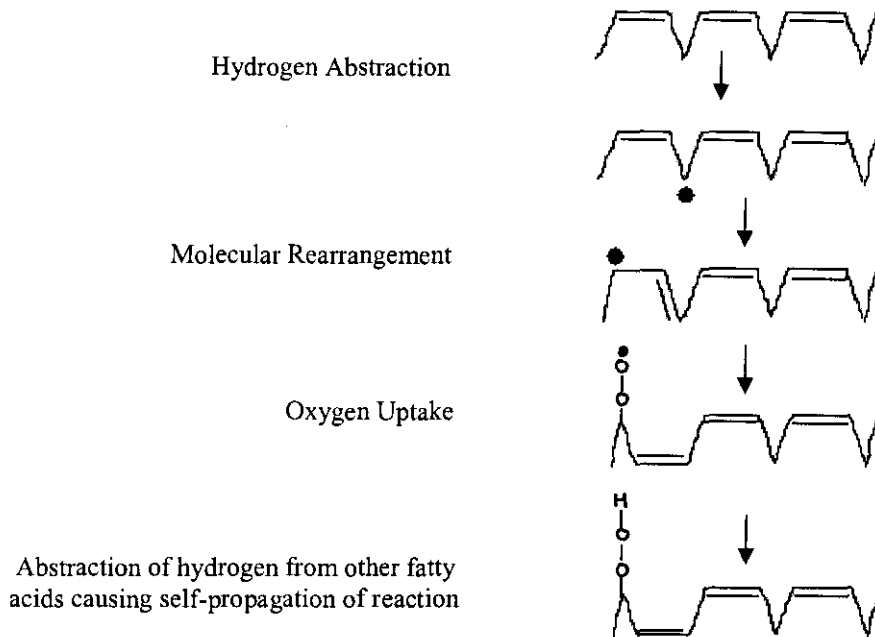
## I. Introduction

Oxidation is an essential biological process for the billions of cells in our body. Oxidation generates energy for the cells to carry out their biological processes. Free radicals, also known as reactive oxidants, are a metabolic by-product of the process of oxidation. These free radicals, all of which contain reactive oxygen species, are the result of normal functioning of the cell, and are released into the body in small amounts. Antioxidant defenses in our bodies include vitamin E, vitamin C, glutathione redox system, catalase, and superoxide dismutase. These defenses readily intercept the free radicals that escape from the cells. However, if the released free radicals exhaust the antioxidant defenses, they remain in the body and are associated with several diseases (Baskin & Salem, 1997).

All molecules have electrons as their outermost constituents. The properties of the molecule are determined by the behavior of these electrons. Electrons have either an upward or downward spin, which generates an electromagnetic field. The effect of this field can be cancelled by pairing two electrons with opposite spins. Thus the most stable configuration of electrons is when two electrons spinning in the opposite direction are paired (Baskin & Salem, 1997). When an electron is unpaired, it is highly reactive and unstable. This reactive species can cause damage to cell structures by stealing electrons from other molecules within the cell, which disrupts normal chemical processes (Baskin & Salem, 1997).

All cells are surrounded by a lipid bilayer, which is partially composed of polyunsaturated fatty acids. The lipids are readily attacked by free radicals, which eventually form lipid peroxides. The oxygen-containing free radicals, generated by normal oxidation but not intercepted by antioxidants, are the major reactive species that can initiate lipid peroxidation. The free radicals abstract a proton from the polyunsaturated fatty acid. The lipid radical then undergoes a reaction with oxygen to form the peroxy radical, which then attacks other polyunsaturated fatty acids in the

membrane. This self-propagation is what makes the free radicals so harmful. When the bilayer is disrupted, that allows things to get into the cell that shouldn't be in there. This process is stopped when the peroxy radicals encounter an antioxidant that breaks the chain reaction (Baskin & Salem, 1997). This process can be seen in the following schematic.



Disease-causing free radicals can also come from non-biological processes. They can be generated by the inhalation of air pollutants or cigarette smoke (Baskin & Salem, 1997). Mine workers frequently develop lung disease. This is related to lipid oxidation because the dust in mines contains asbestos. These asbestos can react with molecular oxygen which generates a species capable of oxidizing many different biological molecules (Favier *et al.*, 1994).

Lipid oxidation in biological systems is the cause of several degenerate diseases. Therefore, there is a lot of interest in the development of methods to determine very small quantities of hydroperoxides, which are the primary products of lipid oxidation (Nakamura &

Maeda, 1991). The optimal method would be relatively simple and fast, have good reproducibility, and high sensitivity.

Triphenylphosphine (TP) has been used for the structural analysis of lipid hydroperoxides for a long time. It reduces hydroperoxides to the corresponding hydroxyl compounds, while being converted to triphenylphosphine oxide (TPO). Therefore, the peroxide content of lipids can be measured by measuring the TPO produced or the TP consumed in the reaction. At low lipid oxidation levels, the determination of trace amount of TP consumed based on the measurements of unreacted TP can lead to substantial errors. Therefore, in the experiment performed by Nakamura *et al.*, the amount of TPO produced was measured using reverse-phase or normal-phase high-performance liquid chromatography (HPLC) along with an ultraviolet (UV)-detector by measuring the absorption at 220 or 260nm. This particular method requires a lot of time, and there are many complications associated with this method (Nakamura & Maeda, 1991).

The first step in this method was to react the TP with hydroperoxide. This required mixing the reagents together and the shaking the mixture at a specific temperature, 30°C, in the dark. A 30 minute reaction time was required for this reaction, and it also required a great amount of materials. Although this method was long and complicated, it had a high sensitivity, being able to measure down to less than 10 picomole. Although this is highly sensitive, the sensitivity isn't as good as the sensitivity obtained with other methods using fluorescence (Nakamura & Maeda, 1991).

A second method of measuring oxidation is by using diphenyl-1-pyrenylphosphine (DPPP). Akasaka *et al.* developed this method because they weren't satisfied with previously developed methods, such as iodometry. Iodometry has mostly been used for the determination of lipid hydroperoxides in foodstuff. The method was too complicated, and its  $10^{-6}$  mol order detection limit wasn't satisfactory. The colorimetric detection of  $I_3^-$  has been used in flow

injection analysis (FIA) for lipid hydroperoxides. The method was fast and had a sensitivity of 100 picomole level. This method is complicated in that it must be tightly capped and left in the dark for 60 minutes at 60°C, the coil must be kept at 80°C during Flow Injection Analysis, there is a six minute lag time, and the method requires that oxygen be removed from solvents (Akasaka & Ohrui, 2000).

A second method that the authors found unsatisfactory was the thiobarbituric acid methods. It was a simple and sensitive method, with a detection limit of  $10^{-10}$  mol order, used to determine oxidation in biological materials. This method measured oxidation as malondialdehyde, instead of as hydroperoxide. This method wasn't used because it had some shortcomings in selectivity and quantitativity because some compounds other than hydroperoxides would be mistaken for hydroperoxides and therefore incorrectly measured (Akasaka & Ohrui, 2000).

Enzymatic methods have also proved difficult. These authors also found FIA methods with the luminol chemiluminescence to be unsatisfactory. They can detect lipid hydroperoxides at the picomole level, but they require the removal of radical scavengers in the sample before analysis because the scavengers satiate the luminol chemiluminescence (Akasaka & Ohrui, 2000).

Since the authors found these methods to be inadequate, they developed a new method of measuring lipid oxidation. They used phosphine reagents to measure hydroperoxides by using batch and flow injection methods, and by HPLC. The best way to measure oxidation was by measuring fluorescence. Phosphines have no fluorescence, but their oxides have very strong fluorescence. When light is shone on the oxides, they shine back with fluorescent light. Measuring fluorescence rather than absorbance is a more sensitive method. In absorbance, light is shone through the sample, and the amount of light absorbed is measured. In fluorescence, the light is shone through the sample, but the light of interest comes out from a different spot. Therefore, with absorbance, there is always a lot of background light, which makes it less sensitive. With

fluorescence, any light that comes out, no matter how small, can be measured, because the background is approximately zero. Therefore, the amount of hydroperoxides is easily determined by measuring the strength of fluorescence intensity of the oxides. The phosphine oxides that contained a 1-pyrenyl group showed stronger fluorescence, therefore diphenyl-1-pyrenylphosphine (DPPP) was found to be the most suitable reagent (Akasaka & Ohrui, 2000).

Total hydroperoxides was first determined by the batch method using DPPP. Lipid hydroperoxides were determined by the increase of fluorescence intensity of DPPP oxide after the reaction. Although the authors were satisfied with this experiment, it was quite a long and complicated process requiring many reagents. The mixture had to be tightly capped, and left for 60 minutes in the dark in a temperature of 60°C. Also, butylhydroxytoluene (BHT) had to be added to the reaction mixture in order to prevent further oxidation after the set reaction time. Following the reaction, the mixture had to be cooled in an ice bath and methanol had to be added to it. Using this procedure, a sensitivity down to 0.1 nanomoles was able to be measured, and with good reproducibility (Akasaka & Ohrui, 2000).

FIA was also used to determine total hydroperoxides. In this process DPPP was used and an HPLC with a post-column system was used for FIA. This procedure was relatively simple, but the mixture had to be reacted in the coil at a specific temperature, 80°C. Using this method, the detection limit was found to be 0.2 picomoles. The results from this method correlated well with the results from the batch method (Akasaka & Ohrui, 2000).

Finally, the same DPPP method was applied to an HPLC post-column system. HPLC was used to separate hydroperoxides and then their amounts of oxidation were measured. The eluent from the column was monitored by a UV detector followed by post-column detection with DPPP. The downside of this process was that there was a six minute lag time on the peaks detected by

fluorometry. With this method, 1-2 picomole levels of hydroperoxides were detected with high selectivity and reproducibility (Akasaka & Ohnishi, 2000).

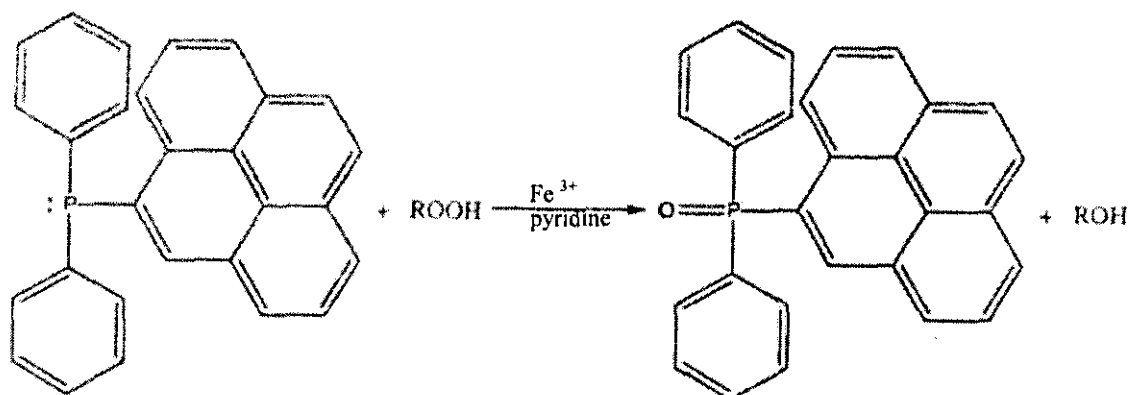
Miyazawa *et al.* developed a third method of measuring lipid oxidation, chemiluminescence detection-high performance liquid chromatography. The researchers were specifically measuring phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide in the liver and brain of a rat. While this method had high reproducibility and sensitivity as low as 10 picomoles, the method and the construction of the calibration curve were rather long and complicated in that the reaction mixture had to be cooled in ice water and photoirradiated for 20 minutes at 10°C. The reaction mixture had to be passed through a silica column with methanol as an eluent to remove methylene blue. The hydroperoxides were recovered in the methanol extract, which then had to be dried under reduced pressure by rotary evaporation. Also, a temperature controlled oven was used, and the post-column mixing joint had to be kept at 40°C (Miyazawa *et al.*, 1992).

The previously described methods weren't sufficient for the measurement of lipid peroxides. They have many complications that make the development of a new method desirable. One of the most significant problems is the time required to run these experiments. It is preferable to have a method that will allow the reaction to finish in a very short amount of time, usually in just seconds. Many of these methods also require a specific temperature, which makes the experiment harder to run. It is also very difficult to run an experiment where the conditions have to be very specific, for example, removing all of the oxygen from solvents or removal of radical scavengers. It is also preferable to have a method that doesn't require a great amount of reagents. Also essential is to have a method that has a high sensitivity.

We therefore sought to develop a simple method that also allowed the reaction to take place in a short amount of time and was highly sensitive. Instead of using actual cells, chemicals



were used that could simulate what occurs in the body. Tertiary butyl hydroperoxide (t-BuOOH) was used to simulate the lipid bilayer. It is a peroxide, so it can imitate a bilayer that has been attacked by a radical and was oxidized. DPPP was used to measure the amount of oxidation. DPPP contains a lone pair, which suppresses fluorescence, but when DPPP reacts with a peroxide, it gains an oxygen, which causes it to lose the lone pair. When DPPP becomes DPPPO, as seen below, it will fluoresce.



Hence, the original amount of oxidation can be measured. The area of the peaks obtained in the chromatogram represent the amount of fluorescence, and therefore the amount DPPP that has been oxidized, which tells how much oxidation was present. The  $\text{Fe}^{3+}$ /pyridine complex is a catalyst that speeds up the reaction between DPPP and t-BuOOH. The exact mechanism behind the  $\text{Fe}^{3+}$ /pyridine catalyst isn't understood, so the goal was to find a good ratio of these two chemicals that would complete the reaction in a short amount of time.

## II. Materials and Methods

A Waters High Performance Liquid Chromatography instrument was used for Flow Injection Analysis. The column mobile phase was a 1:1 volume ratio of HPLC grade chloroform and HPLC grade methanol. The flow rate was 1 ml/min. Reagents were mixed together and aliquots were injected to make a baseline. Following the baseline measurement, the final reagent

was added, and aliquots of the mixture were injected. The column eluent passed through to a detector set at an emission wavelength of 380nm and an excitation wavelength of 352 nm.

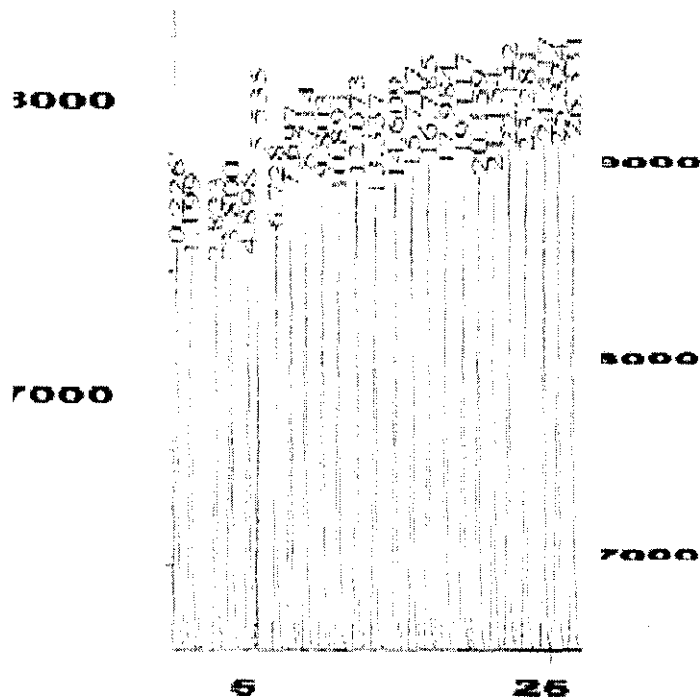
A baseline was measured before adding the t-BuOOH. The baseline solution consisted of varying amounts of CHCl<sub>3</sub>:MeOH, 0.01mg/ml DPPP in CHCl<sub>3</sub>:MeOH, 0.01M Fe<sup>3+</sup> in MeOH, and 12.4 M pyridine. Three to four 6μl injections of pure CHCl<sub>3</sub>:MeOH were injected to clean the column prior to use. Following the cleaning, 3-4 6μl injections of the reaction mixture without t-BuOOH were made in order to get a level baseline. Subsequent to the baseline injections, 10 μl of a 7 x 10<sup>-6</sup>M solution of t-BuOOH was added. Within 30 seconds of adding the t-BuOOH, 6 μl aliquots of the reaction mixture with the added t-BuOOH were injected. All parts of this experiment were carried out at room temperature. Approximately 20 injections were done.

### III. Results

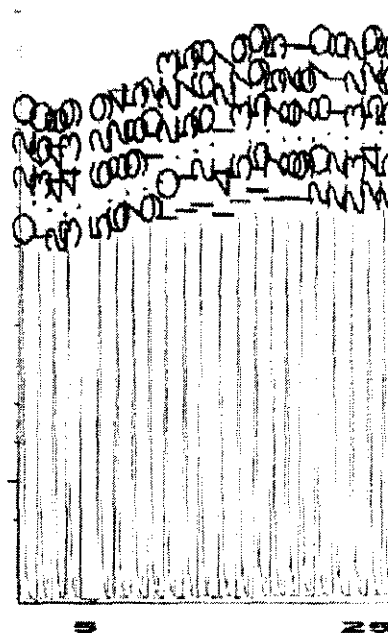
For every experiment run, the total volume of the reagents used to make each reaction mixture was 1000 μL. In order to determine the best amount of each reagent to use, the amounts of different reagents were varied each time. The data was then analyzed and used in helping to determine what amounts needed to change. The amount of pyridine used was what was most frequently changed throughout the experiments, but when the volume of pyridine used was changed, the volume of CHCl<sub>3</sub>:MeOH was also changed to keep the total volume at 1000μL. For each experiment, the chromatograms were studied and graphs were made to determine the final slow reaction rate. Our ultimate goal was a slope of zero. Throughout all the experiments, after getting a steady baseline, 10 μL of a 7.0 x 10<sup>-6</sup> M solution of t-BuOOH was injected into the solution. In the earliest experiments, the initial reaction mixture consisted of 150 μL of 12.4M pyridine, 150 μL 0.01M Fe<sup>3+</sup> in MeOH, 40 μL 0.01mg/mL DPPP in CHCl<sub>3</sub>:MeOH, and 710 μL CHCl<sub>3</sub>:MeOH. The chromatogram shown in graph 1 is a representative graph from the HPLC instrument of the all of the experiments done using 150 μL of a 12.4M solution of pyridine.

Graph 2 is a chromatogram from an experiment using 325  $\mu\text{L}$  of 12.4M pyridine.

Graph 1:

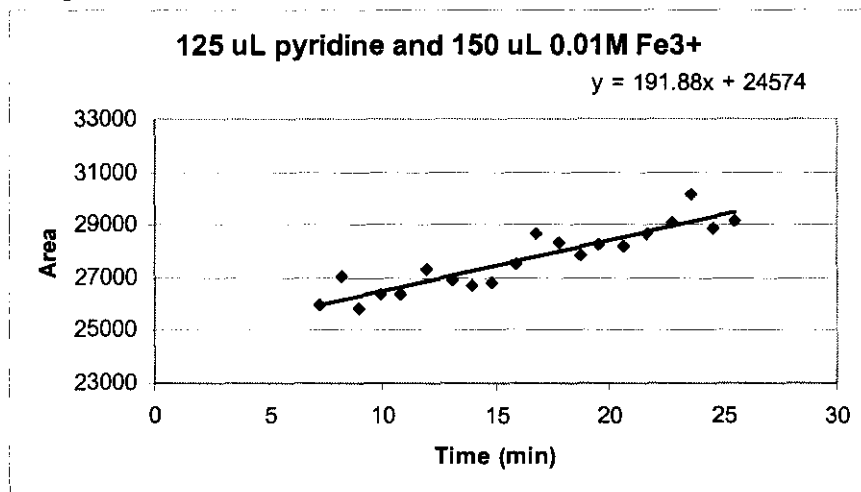


Graph 2:

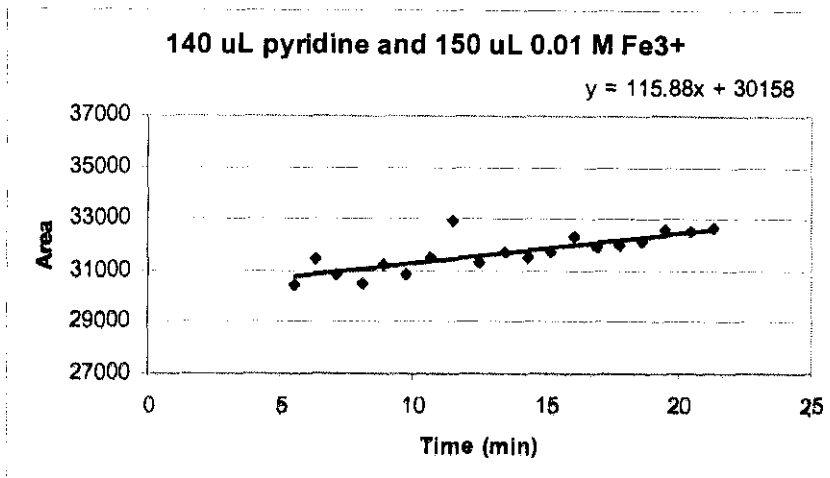


Graphs 3-11 plot the peak area versus time to compare the slopes of experiments using 125 $\mu\text{L}$ , 140 $\mu\text{L}$ , 150 $\mu\text{L}$ , 160 $\mu\text{L}$ , 200 $\mu\text{L}$ , 300 $\mu\text{L}$ , 325 $\mu\text{L}$ , 375 $\mu\text{L}$ , and 425 $\mu\text{L}$  of pyridine.

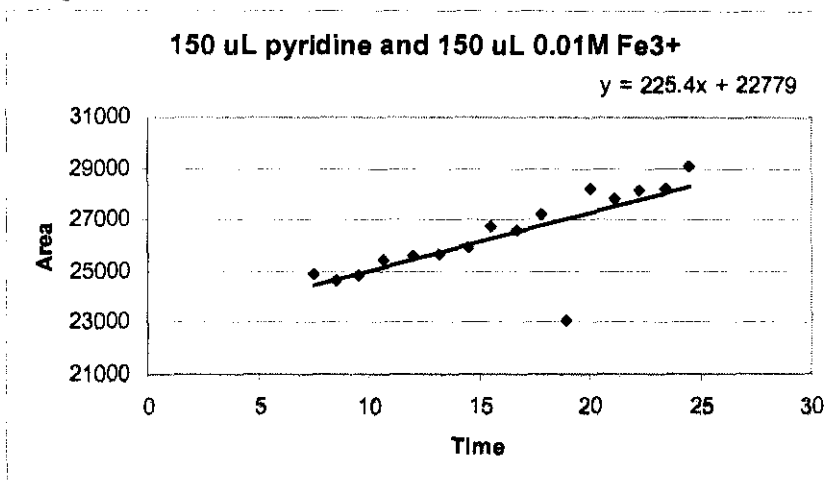
Graph 3:



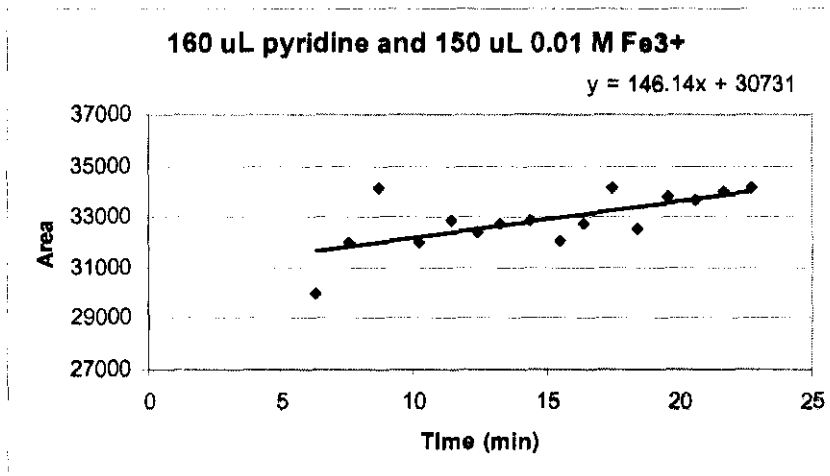
Graph 4:



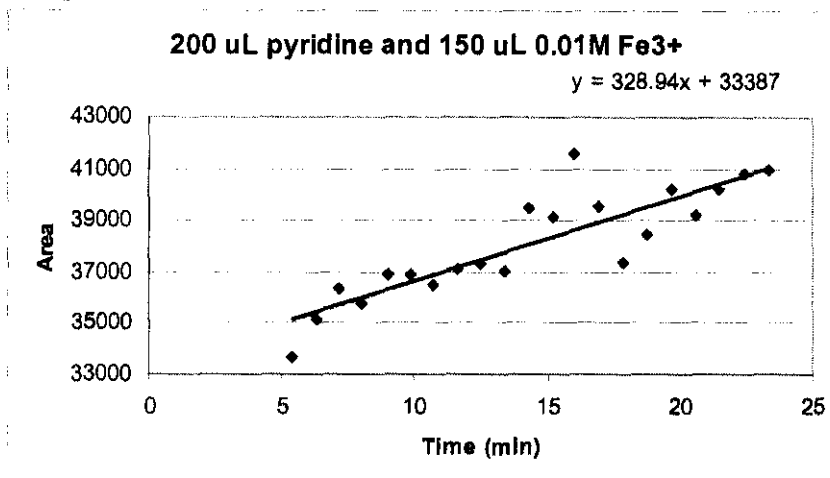
Graph 5:



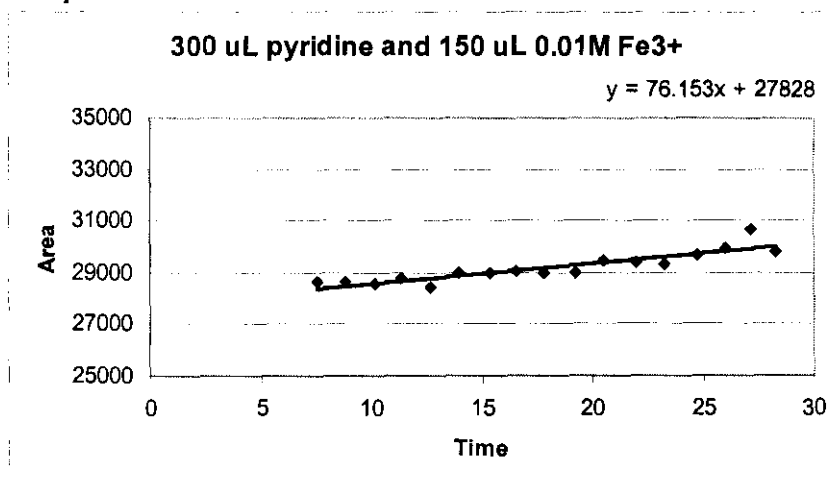
Graph 6:



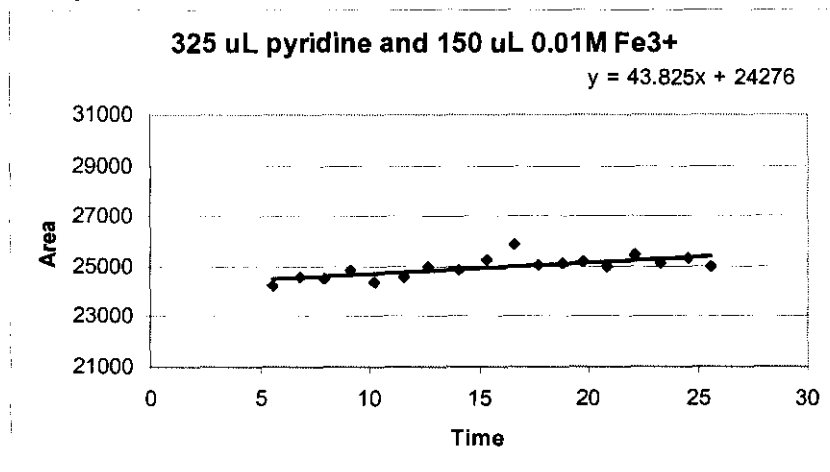
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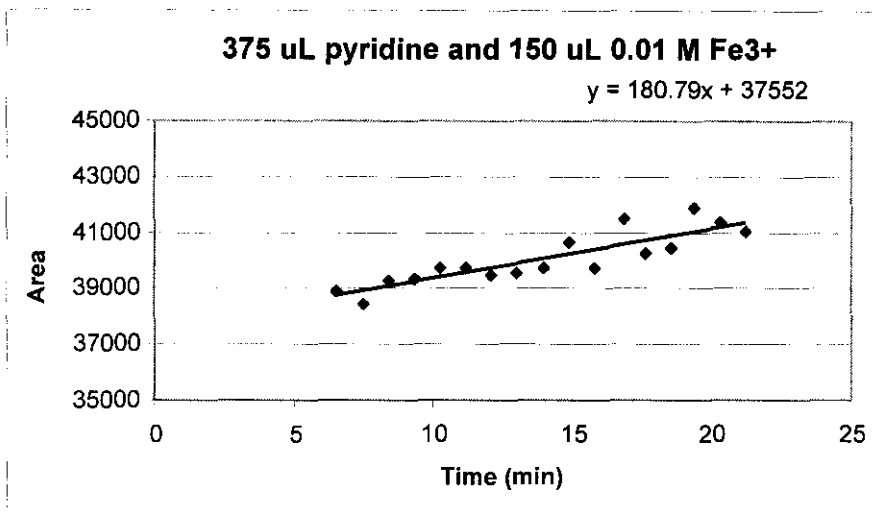
Graph 8:



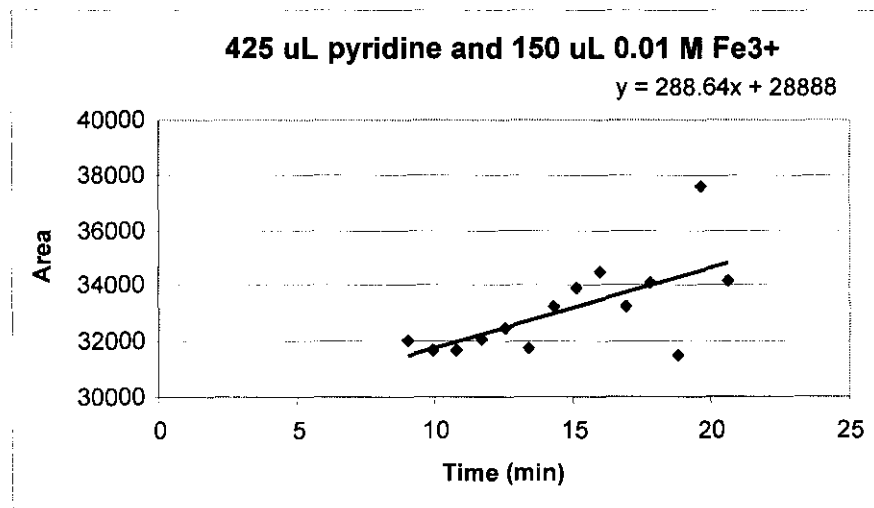
Graph 9:



Graph 10:

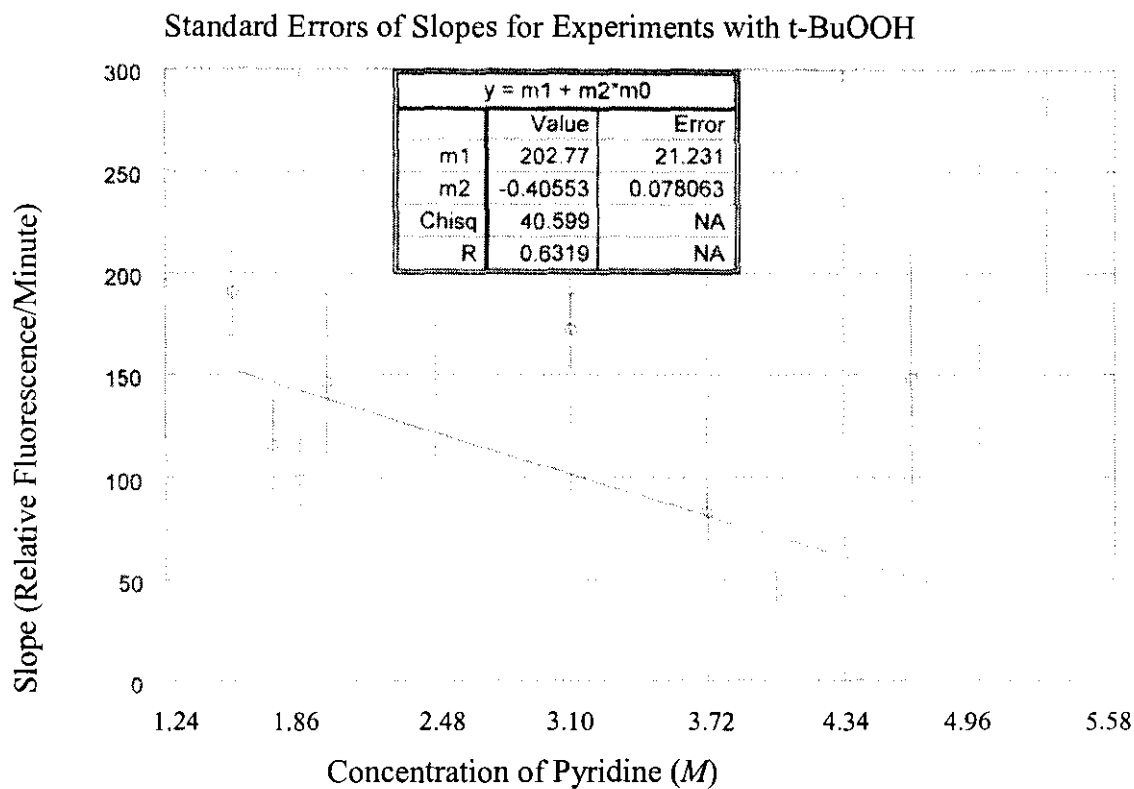


Graph 11:



Graph 12 shows the final reaction rate after adding  $7 \times 10^{-6} M$  t-BuOOH. It also includes the standard errors for each concentration.

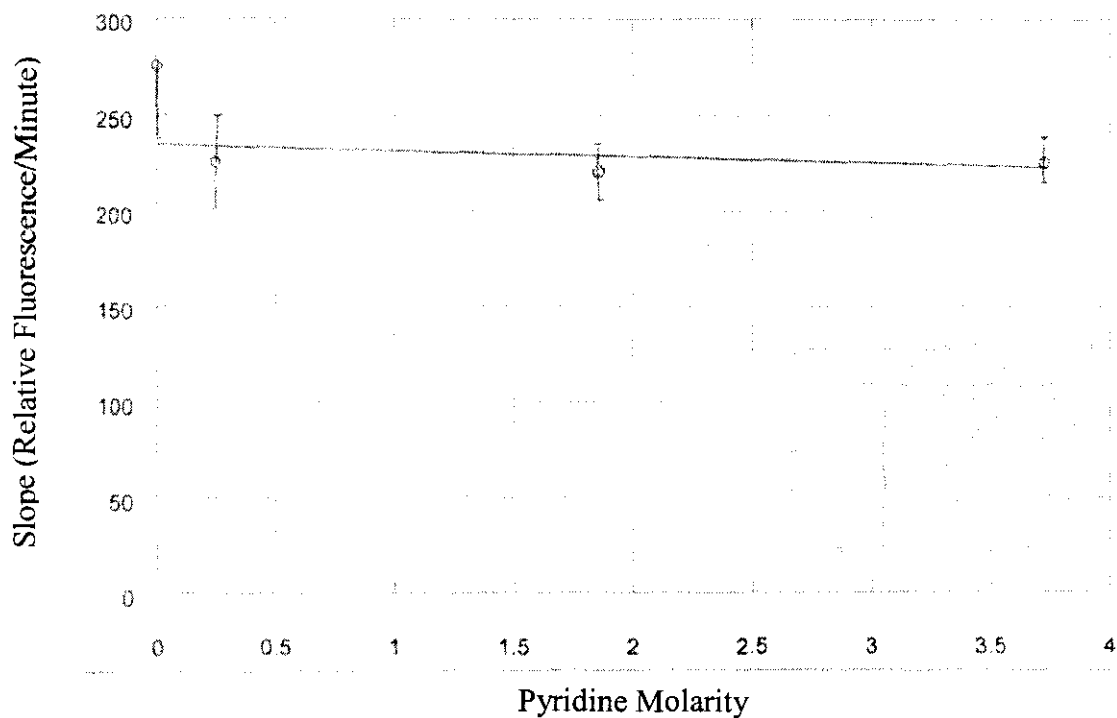
Graph 12:



Graph 13 plots the same variables as graph 12, but for experiments in which no t-BuOOH was added. The standard errors are also included.

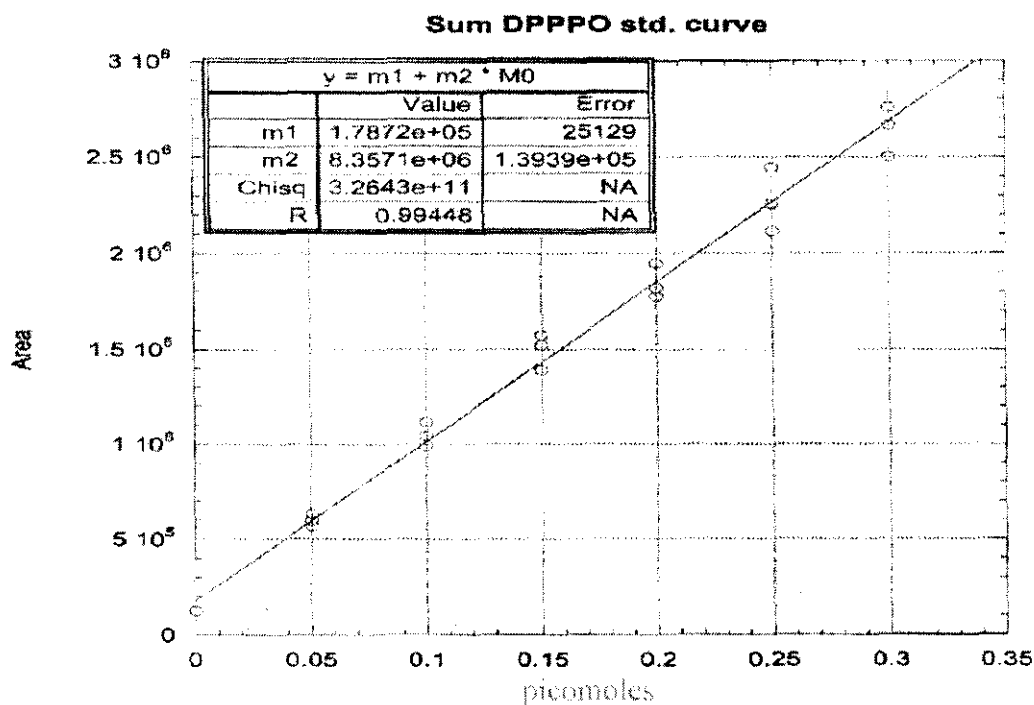
Graph 13:

Standard Errors of Slopes for Experiments in which no t-BuOOH was added



Graph 14 shows the sensitivity of the method being used.

Graph 14:





#### IV. Discussion

The progress of the experiment was monitored by measuring the peak areas on the chromatograms. Peak areas are an indication of fluorescence. When DPPP reacts with the hydroperoxide, it becomes DPPPO and will then fluoresce. Steady peak areas are an indication of a completed reaction, because that means no more DPPP is reacting, and all the hydroperoxide has reacted. A set of peaks in which the areas are increasing indicates that as time progresses, more DPPP is being converted into DPPPO, and the reaction has not yet finished. The goal is to run an experiment that produces steady peak areas, meaning that the reaction takes place, and is completed in a very short amount of time. Making graphs of time versus amount of fluorescence for differing amounts of pyridine, the catalyst, is an effective way monitor the progress of the reaction. By making graphs, the slope can be determined. A graph with a large slope indicates that the reaction is still progressing. Therefore a lower slope value indicates that the reaction is nearing completion, and a slope of zero would be optimal. As is discussed later, it is possible that the reaction of DPPP with the t-BuOOH has completed even when the areas continue to increase. Pyridine could dissolve a lot of molecular oxygen, and therefore the increase in peak areas could be due to the reaction with molecular oxygen.

The first couple of peaks shown in the chromatograms are baseline peaks, or peaks that measure the amount of DPPPO present before the tBuOOH is added. DPPPO is present even before the tBuOOH is added due to DPPP reacting with molecular oxygen. The reaction with molecular oxygen is what causes baseline peaks to be necessary. Before the reaction of interest begins, some DPPPO is already present in the solution. This isn't important; what is important is the increase in peak area after the addition of t-BuOOH. Therefore the original amount of DPPPO is taken into account when the baseline peaks are made, which are like a zero point. In the experiments, DPPP is in great excess, so having some of it react with molecular oxygen before the

experiment doesn't affect the results of the experiment. Also important is that DPPP becoming DPPPO is a one way reaction, so the reverse reaction doesn't have to be taken into account when analyzing the change in peak area. It would probably be beneficial to use a lower concentration of DPPP in the reaction mixture, because it is in such great excess. By using less DPPP, there would be less of a reaction with molecular oxygen.

The initial jump occurs after the t-BuOOH is added, when a lot of DPPP quickly reacts and becomes DPPPO. As can be seen in graph 1, a graph of an experiment using 150 $\mu$ L of pyridine, throughout the 25 minutes that the experiment is run, the peaks continue to increase sharply, indicating that the reaction has not neared completion. As graph 2 shows, the peaks are much steadier with 325 $\mu$ L pyridine. Although the peaks in these two graphs look different, when the standard error is considered, as is discussed later, the reaction is probably taking place at the same rate in both of these experiments. It can be concluded that the reaction nears completion in a relatively short amount of time.

Graph 12 is graph plotting pyridine concentration versus slope with standard errors for each of the experiments. The ideal experiment would have slope of zero. By looking at this graph, it appears that there is no obvious pattern to the results. It looks as if the slopes follow no logic that would lead to knowing the correct amount of pyridine. Therefore, the volume of each reagent used in each experiment run in this research was determined by looking at the results of previous experiments and trying to determine which volumes should be increased and which should be decreased. From this graph, it becomes apparent that when the standard errors are considered, there is no significant slope difference when the concentration of pyridine is varied.

$\text{Fe}^{3+}$  and pyridine form a complex that acts as a catalyst that speeds up the reaction between DPPP and the hydroperoxide. This catalyst is the key to getting the reaction completed in a short amount of time. Very little is understood about the  $\text{Fe}^{3+}$ /pyridine catalyst, which is why

working with this catalyst involves so much guessing. Derek Barton, a researcher at Texas A & M University, has found in his experiments that the  $\text{Fe}^{3+}$ /pyridine complex is an effective catalyst. He was oxidizing triphenylphosphine to its oxide in pyridine and found that the presence of  $\text{Fe}^{3+}$  greatly increased the rate of the reaction. He found that the reaction was more than 40 times faster in the presence of the catalyst (Barton *et al.*, 1997).

The rates of the reactions were closely studied in this research. The  $\text{Fe}^{3+}$ /pyridine catalyst greatly affects the rate, and therefore the amount of it used in these experiments was very important. In most of the experiments performed,  $0.0015\text{M Fe}^{3+}$  was used. This was because since pyridine and  $\text{Fe}^{3+}$  form a complex, changing the amount of pyridine can accomplish the same thing as changing the amount of  $\text{Fe}^{3+}$ , and it is much easier to compare results if only one variable is changed and the rest are kept constant. Since the  $\text{Fe}^{3+}$  and the pyridine form a complex, it would be helpful to know exactly the amount of  $\text{Fe}^{3+}$  and pyridine that bind with each other. Up to  $5.27\text{M}$  pyridine has been experimented with. When taking into consideration the concentrations of  $\text{Fe}^{3+}$  and pyridine used, it seems probable that the  $\text{Fe}^{3+}$  is saturated with pyridine at all pyridine concentrations and therefore increasing the pyridine isn't going to have a beneficial effect on the rate of the reaction.

At high pyridine concentrations, there was initial concern that the pyridine was just suppressing the signal. This would mean that the reaction hadn't actually completed, but that there was just so much pyridine present that the signal was suppressed, and the reaction appeared to be completed. This concern can be ruled out just by looking at peak areas. Using  $425\mu\text{L}$  of pyridine suggested that this was not a concern, because even with this much pyridine, the signal was not suppressed and the peak areas were the same size as for low amounts of pyridine. This therefore allowed us to know that the signal wasn't being suppressed because it could still be seen that a reaction was still taking place. Using this much pyridine also raises another question. With

this high of an amount of pyridine, it becomes the solvent, instead of  $\text{CHCl}_3\text{:MeOH}$ . With this amount of pyridine, it could be dissolving a lot of molecular oxygen, and therefore the increase in peak areas could be due to the reaction with molecular oxygen, and not with t-BuOOH. In order to confirm that a reaction between DPPP and molecular oxygen wasn't significant, an experiment was run with no t-BuOOH to see what effect this would have.

In all of the reactions carried out, the slope calculated was positive. This confirms that a reaction was still taking place. What this information doesn't disclose is what that reaction is. It takes approximately 30 seconds to inject the solution for Flow Injection Analysis after adding the t-BuOOH to the reaction mixture. Thus, what happens during that 30 seconds isn't able to be monitored. There are two possible things that could be happening during these reaction experiments. DPPP is in great excess of t-BuOOH, therefore the rate depends on t-BuOOH. When the slope of one of these experiments is monitored, it is found that the slope is a large positive number, indicating that a reaction is still taking place. The first possibility is that one reaction is taking place, between DPPP and t-BuOOH, and since the slope is increasing, the reaction between DPPP and t-BuOOH doesn't complete during the time that the reaction is monitored. Since the first 30 seconds of the reaction isn't able to be monitored, all that is seen is a linear line with a positive slope. A second possibility is that there are two reactions taking place. One reaction is between the DPPP and t-BuOOH. This reaction would have a large positive slope at the beginning when the reaction is taking place, but would then quickly level out after all the t-BuOOH had reacted. The second reaction would be between DPPP and molecular oxygen. The line representing this reaction would be linear, and slowly increasing. Since the two reactions would be taking place at the same time, the actual line seen would be linear with a positive slope. Since the beginnings of the experiment can't be seen, the only line that is able to be seen is after the 30 seconds and would therefore appear the same for both the reaction possibilities and be a

straight line with a positive slope. Another set of experiments had to be done to determine if one reaction was taking place or if two were taking place.

In order to see which of the two reaction possibilities was actually taking place, a set of experiments were run without any t-BuOOH. Without any t-BuOOH, the only thing for DPPP to react with would be molecular oxygen. The usual reaction mixture was used, and the amount of pyridine was varied. Experiments were run with 0M, 0.00062M, 1.86M, and 3.72M pyridine. In each of these experiments, the slope was approximately the same, with a value close to 250 (relative fluorescence/minute). A graph, including standard errors can be seen in graph 13.

From this data, it can be concluded that two reactions are taking place in the experiments when t-BuOOH is used. The reaction with t-BuOOH is quickly completed, and the positive slope seen in all of the previous experiments is due to the reaction between DPPP and molecular oxygen.

Since the  $\text{Fe}^{3+}$  and pyridine aren't understood very well, it is not known what kind of an effect these catalysts have on reaction when they are not bound. After the  $\text{Fe}^{3+}$ /pyridine complexes, are used up, maybe the  $\text{Fe}^{3+}$  or the pyridine, whichever is still left, catalyzes the reaction on its own. If this is the case, they probably catalyze it more slowly on their own than when they are complexed. Since the concentration of pyridine is so large compared to the concentration of  $\text{Fe}^{3+}$ , it would probably be pyridine that would be left to catalyze the reaction on its own. If this was the case, the graph of concentration of pyridine versus slope should have a positive slope. The graph, which is graph 12, was relatively scattered, but when the standard errors are taken into account, the slope of the lines is near zero.

Graph 14 illustrates the sensitivity of the method being used. As the graph shows, this method is extremely sensitive. Amounts down to 0.05 picomoles can be measured.

Free radicals play a role in several diseases, including cancer and cardiovascular disease. The lipid hydroperoxides generated by free radical attack are promptly decomposed by traces of transition metal ions to produce the free radical intermediates of lipid peroxidation capable of propagating the chain reaction. Reactive oxidant species-mediated damage to DNA bases is involved in mutation and the development of tumors. Oxidative DNA damage involves the modification of DNA bases. Mutations can also result from oxidative damage to the membranes. The lipid peroxides formed as a result of this oxidative membrane damage can then decompose to mutagenic carbonyl products. Lipid peroxidation may play a role in breast cancer risk. Studies have found that the mutagen malondialdehyde in urine of women is twice as high in women with a high risk of cancer as in women with a low risk of cancer (Wiseman *et al.*, 2000).

Lipid oxidation is thought to be primarily responsible for the aging process. It is believed that the detrimental actions of reactive oxidant species are to blame for the functional deterioration found with ageing (Wiseman *et al.*, 2000).

Oxidative damage to mitochondria has been connected to neurodegenerative disorders such as Parkinson's disease. Radicals formed during lipid peroxidation can harm mitochondrial DNA by causing mutations and deletions. These damages accumulate with age, and are also involved in the development of Alzheimer's disease (Wiseman *et al.*, 2000).

Membranes play an extremely important role in the function of cells. They have a structural role, but they also participate actively in chemical reactions such as the transmission of impulses by brain cells. An important factor for the viability of a cell is the fluidity of its membrane. As humans age, the fluidity of cell membranes usually decrease, as a result of increased cholesterol content. Many laboratories have found platelet membranes of patients with Alzheimer's disease to be more fluid than normal. In most cases, increased membrane cholesterol is normally not desirable, since it is associated with decreased learning ability. When membranes

are damaged by lipid peroxidation, specific enzymes are responsible for incorporating cholesterol into cell membranes in order to repair them (Van Rensburg *et al.*, 1994).

Some researchers have suggested that an irregularity of the endoplasmic reticulum may be the cause of the increased fluidity. The microviscosity of internal endoplasmic reticulum was studied. The lipid composition of the endoplasmic reticulum differs from the external membrane in that the endoplasmic reticulum membranes contain very little cholesterol. Thus, unlike the external membrane, they probably don't have the enzyme necessary for making cholesterol. Therefore, these two membranes would respond differently to lipid peroxidation; external membranes would be able to produce cholesterol to protect itself against the damage, while the endoplasmic reticulum would not be able to protect itself. X-ray diffraction analysis of the membranes of Alzheimer's patients has found a reduction in the lipid bilayer width in membranes due to a cholesterol deficit in these membranes (Van Rensburg *et al.*, 1994).

It has been discovered that the genetic variant transferrin C2, whose function it is to bind aluminum and iron, connects free radicals to Alzheimer's disease. Transferrin C2 is recurrently found in Alzheimer's disease patients. This brought about an investigation into the effect of lipid peroxidation caused by hydroxyl radicals on platelet membrane fluidity. The iron binding capacity of transferrin C2 has been shown to be low, which would cause the iron carried by this transferrin variant to be more available for free radical reactions (Van Rensburg *et al.*, 1994).

The peroxidation of the membrane is thought to occur in the following way. Iron attached to transferrin is transported into cells by the process of endocytosis. The pH inside the endocytic vesicles is low, which causes the release of iron which is then bound by a ligand and transported into the cell for utilization or storage in ferritin. If the transfer of iron to the ligand were impaired due to genetically defective transferrin, the iron could stay in the solution long enough to be reduced by an electron donor, and lipid peroxidation would then be initiated. One mechanism by

which lipid peroxidation can be started in a biological system is by the production of hydroxyl radicals, which requires the simultaneous presence of a transition metal ion, hydrogen peroxide, and superoxide, which is the reducing agent (Van Rensburg *et al.*, 1994).

Another consequence of the accumulation of free radical damage is the development of cataracts. In studying the causes of membrane damage in eye lenses showed increased formation of lipid peroxidation products, which suggests that the damage to the lens membrane plays a direct role in the damage caused overall (Wiseman *et al.*, 2000). Lipid peroxidation has also been found to be the cause of several digestive diseases (Yagi, 1998). The best protection against all of these diseases is the consumption of dietary membrane antioxidants, many of which are found in fruits and vegetables (Wiseman *et al.*, 2000).

As it can be seen, free radicals and the lipid oxidation that they cause are very closely linked to human health. In learning about these diseases, the need for a more sensitive assay becomes apparent. Before these diseases can be treated, the mechanisms behind them need to be understood more clearly. By having a sensitive assay for measuring lipid oxidation, it will be possible to determine how much of a role lipid oxidation plays in these diseases and how much oxidation has to have occurred in order to have a bad effect on the body.

The method we developed is effective for measuring lipid oxidation. It is a simple method, with few reagents, and the solutions are easy to prepare. The method is fast, because no waiting is involved, and the reaction that takes place happens very quickly. No special conditions are required for this method, so it is easily carried out. As was discussed previously, it has a very good sensitivity, being able to measure down to 0.05 picomoles. The  $\text{Fe}^{3+}$ /pyridine catalyst was found to be very effective, and amounts of both catalysts in the complex were found that give accurate results and allow the reaction to complete in a shorter amount of time. Additional experiments also ruled out potential problems. These experiments confirmed that pyridine doesn't



depress the signal, and that the increase in peak area is due to a reaction with molecular oxygen, not with t-BuOOH. Overall, an effective, reliable method was developed that should be beneficial in further research.

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